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Heavy metal accumulation by *Eisenia fetida* and its effects on Glutathione-S-Transferase activity

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Summary. Bioaccumulation of cadmium, zinc and lead by *Eisenia fetida typica* exposed to five contaminated field soils was studied. Worms regulate zinc burdens but not cadmium or lead. No linear regression was found relating worm cadmium burdens to total amount in soils, most likely due to differences of bioavailability. Ammonium acetate, calcium chloride and water extraction techniques of heavy metals from soils were tested and related to worm burdens. Except for zinc, which is more closely related to less drastic extraction techniques, total soil content of metal seems to be the most interesting to relate worm heavy metal burdens. Moreover, the effects of cadmium, zinc, lead, and copper chloride on worm glutathione-S-transferase activities were studied in vitro and in vivo. GST activity appears not to be affected by heavy metal bioaccumulation as no statistically significant effects were observed neither in vivo nor in vitro on total GST activity.

Key words: Heavy-metals, bioaccumulation, bioavailability, *Eisenia fetida*, glutathione-S-transferase

Introduction

Metal pollution may disturb soil ecosystems by affecting the structure of soil invertebrate populations. As earthworms are very well represented in soil, and as they play an important role in soil bioturbation and degradation of organic litter, a great deal of attention has been paid to their susceptibility to heavy metals. Laboratory and field studies regarding metal uptake by earthworms have shown that some metals such as cadmium, lead, zinc and copper are substantially concentrated in worms tissues (Scaps et al. 1997; Neuhauser et al. 1995; Spurgeon & Hopkin 1996b; Marinussen et al. 1997). Bioconcentration of heavy metals in worms depends on soil metal burdens but also on their bioavailabilities (Spurgeon & Hopkin 1995, 1996b). Generally, it has been found that various factors such as clay, organic matter content or pH influence the bioconcentration of metals (Abdul Rida & Bouché 1997; Spurgeon & Hopkin 1996b).

Another observation made in the literature concerns the lack of worms in highly contaminated fields (Spurgeon & Hopkin 1995). Several studies in artificially contaminated soil suggest that growth and reproduction performances are affected by heavy metals (Reinecke &

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Reinecke 1996; Spurgeon & Hopkin 1996a). Nevertheless, little is known about effects of heavy metals on worm detoxification enzymes. Scaps et al. (1997) showed no observable effect of cadmium or lead on cholinesterase or on other metabolic enzyme pathways, but Goven et al. (1994) showed a negative effect of copper on lysosyme activity.

In vertebrates, since glutathione seems to serve as a first line of defence against heavy metal cytotoxicity prior to induction of metallothioneins (Susuki et al. 1996), several studies on the effects of metals on glutathione-S-transferases (GST) have been conducted. GST may play an important role in association with metallothionein-like lead-bound protein and zinc metallothioneins in the detoxification of lead (Susuki et al. 1996).

In invertebrates, Almar et al. (1987), using the red swamp crayfish, *Procambarus clarkii*, found that cadmium exposure reduces GST activity in the hepatopancreas. Thaker and Haritos (1989) reported that cadmium exposure affects enzyme activity in the hepatopancreas of the shrimp *Callianassa tyrrhena*, producing in vivo increases in glutathione-S-transferase activity and esterase activity. In a species of crayfish *Astacus astacus*, it was found that after cadmium or lead exposure, the activity of three enzymes in the gills and hepatopancreas, namely succinic dehydrogenase, NADPH-cytochrome P450 reductase, glutathione-S-transferase, decreased (Meyer et al. 1991). Since glutathione-S-transferase seems to be an important group of enzymes which have a high activity in worms (Stenersen 1984), the effects of cadmium, lead, copper and zinc on GST activities of worms were studied.

In this paper, bioconcentrations of cadmium, zinc and lead by *Eisenia fetida* exposed to five contaminated field soils, collected in the vicinity of a zinc and lead smelter, were measured. To further investigate the relationships between bioconcentration and bioavailability of metals, total ammonium acetate and calcium chloride extractable metal contents of the five types of soils were measured. After six weeks of exposure, GST activity of the contaminated worms was then measured. In vitro activity of enzyme under heavy metals conditions were also tested.

Materials and Methods

Soil analysis

Five contaminated sites in the vicinity of a lead and zinc smelting works (located at Noyelles-Godault, between Lens and Douai, Northern France) were chosen according to their distance and exposure to wind (IA: 1.1 km N; IB: 0.75 km N; IC: 0.75 km N/NW; II: 0.75 km NW; III: 2.5 km E/SE). Soil was collected from the top 10 cm layer after removing surface vegetation. Prior to analysis, samples were oven dried (80 °C) to constant weight, pulverized and sieved (2 mm mesh size). Samples were treated as described in Scaps et al. (1997) with an adapted normalized method (NF X 31-151, AFNOR 1994) being digested with concentrated nitric and hydrochloric acid. The potentially available fractions of metals in soil were determined according to the NF X 31-120 (AFNOR 1994) after being extracted with ammonium acetate and EDTA. Fractions extractable by $CaCl_2$ (0.01 M) were measured at the «*Laboratoire d'Analyses des Sols* of I.N.R.A. d'Arras». Concentrations of cadmium, lead or zinc in soil or animal samples were determined by flame atomic absorption spectrophotometry (Varian Spectr-AA-10) using an air acetylene flame with deuterium lamp background correction. The same samples were also analysed by ICP AES (Varian Liberty Serie II, data not shown), giving comparable results for the three metals studied. Soil pH was measured on water extracts. Assessment of carbonates was made by HCl digestion in a closed vessel according to the NF X 31-105 method (AFNOR 1994). The volume of carbon dioxide which is produced is measured. Carbonate contents (% dry weight) were then determined with respect to a calcium carbonate calibration curve.

Determination of organic carbon was performed with an adapted sulphochromic oxidation (HACH 1989). Absorbance was measured at 610 nm with a HACH DR/2000 spectrophotometer. In order to determine total nitrogen content, a sulphuric acid and hydrogen peroxide digestion (Digestdahl digestion apparatus-HACH 32130-20) of soils was performed. Digested samples were then treated as described in HACH (1989).

Animals analysis

In all experiments we used *Eisenia fetida typica*. Ten adult specimens of *Eisenia fetida typica* were maintained in each exposure tank containing 200 g of contaminated soil. Three replicates were used for each designated sample of soil and the experiment lasted 6 weeks. Worms in each container were fed metal-free lime foliage and distilled water was added to give a moisture content of 30 % wet weight. Controls were established under in the same condition but in metal-free commercial mould (coniferous bark and peat moss compost). After the exposure, earthworms were removed from their containers, rinsed in tap water and transferred to moist filter paper to void the gut content.

After two periods of exposure (21 days and 31 days), earthworms were digested as previously described (Scaps et al. 1997). Enzyme activity was measured after 6 weeks of exposure. Homogenates of worms were made in 5 ml Tris HCl (20 mM, pH 7.4, 4 °C) buffer, centrifuged for 15 min at 2800 g (Beckman J2-21M/E) and the supernatants were frozen at –20 °C. Before use, aliquots were centrifuged again for 30 min (4 °C) at 17600 g (Sigma 2k15). Protein content and GST activity of the supernatant were determined according to the methods of Bradford (1976) and Habig et al. (1974) respectively, adapted for a microplate reader (Dynatech MR 5000). For protein content measurements, Bio-rad protein reagent was added to diluted tissue extract as described by Scaps et al. (1997), absorbance was read at 610 nm and the protein content of the sample determined with respect to a globulin calibration curve. GST activity was assayed in 250 µl of 50 mM Tris HCl buffer (pH 7.4) with CDNB as electrophilic substrate (1 mM final) and GSH (1 mM final) as the second substrate.

In order to test the effects of heavy metals on GST activities in vitro, $ZnCl_2$, $CuCl_2$, $CdCl_2$, and $PbCl_2$ were added separately to samples of worm homogenates. Final concentrations of 200 µM $ZnCl_2$, 200 µM $CuCl_2$, 200 µM $CdCl_2$, 40 µM $PbCl_2$ were separately obtained and tested as described previously. Worms living in an artificially contaminated environment (80 mg/kg of $CdCl_2$ in soil) for two years were also analysed. All measurements for each condition were made in four replicates.

Results

Soil analysis

The levels of ammonium acetate-EDTA (NH_4Ac), calcium chloride ($CaCl_2$) extracted and total or nitric acid extractable (NAX), zinc, cadmium and lead for all contaminated field soils are shown in Table 1. A very high standard deviation for lead NAX content of site II soils (513 ± 390) can be explained by a very high heterogeneity of the samples (240 µg/g to 2269 µg/g dry weight). This phenomenon was not observed for zinc, suggesting a subsequent lead pollution type of certain samples. NH_4Ac extracted heavy metals are very significantly correlated to total (NAX) content (*r* test: $P < 0.001$) for all the three metals: $y = 0.884\ x - 258$, $r^2 = 0.97$ for lead, $y = 0.577\ x + 0.86$, $r^2 = 0.99$ for cadmium, $y = 0.329\ x - 32$, $r^2 = 0.95$ for zinc. NH_4Ac extracted heavy metal contents were approximately proportional to NAX contents. The percentage of extraction represented by the slope parameter is about to 88 % for lead, 57.7 % for cadmium and 32.9 % for zinc. The only correlation found was between NAX and NH_4Ac extracted metals. No correlation was found with other tested parameters (organic matter content: OM %, calcium carbonate content: $CaCO_3$ %, nitrogen content: NTK).

Concentration of metals in exposed earthworms

No mortality of *Eisenia fetida* occurred in the contaminated soil exposure tanks. The uptake of cadmium, zinc and lead is shown in Table 2. For cadmium and lead, the bioconcentration (ratio of metal content weight to total dry tissues weight) clearly increased with exposure time. Bioaccumulation factors (ratio of bioconcentration to total soil concentration) were calculated for each soil type after 31 days of exposure. These accumulation factors for cadmium are very variable between soil types: 0.67 for IB-soil (the most contaminated, Table 1) to 3.18 for IC-soil. Except for IB-soil, bioconcentration factors are higher than one.

Table 1. Heavy metal contents (µg/g dry weight) of soil collected in the vicinity of a zinc and lead smelter located at Noyelles-Godault, between Lens and Douai (Nord-Pas de Calais, France). All values are the average of at least three replicate samples. Values in brackets give the SD.
(NH_4Ac): ammonium acetate extractable heavy metals. (NAX): nitric acid extractable heavy metals. ($CaCl_2$): calcium chloride extractable heavy metals. OM (%): Organic matter content. $CaCO_3$ (%): Calcium carbonate content. NTK: Kjeldahl extractable nitrogen content

Sample	pH	OM *(%)*	$CaCO_3$ *(%)*	NTK *mg g^{-1} dry wt*	Cd *(NAX)* *µg g^{-1} dry wt*	Pb *(NAX)* *µg g^{-1} dry wt*	Zn *(NAX)* *µg g^{-1} dry wt*	Cd *(NH_4Ac)* *µg g^{-1} dry wt*	Pb *(NH_4Ac)* *µg g^{-1} dry wt*	Zn *(NH_4Ac)* *µg g^{-1} dry wt*	Cd *($CaCl_2$)* *µg g^{-1} dry wt*	Pb *($CaCl_2$)* *µg g^{-1} dry wt*	Zn *($CaCl_2$)* *µg g^{-1} dry wt*
IA	7.3	2	0.7	1.8 (0.04)	13 (2.83)	798 (117.7)	973 (37.16)	5.5 (0.091)	538 (46.2)	207 (44.0)	0.21	<0.2	1.2
IB	7.3	11.6	0.3	5.35 (0.28)	89 (2.58)	4204 (529.45)	5581 (627.5)	52 (1)	3576 (178.2)	1860 (72.1)	5.05	2.6	159.70
IC	7.5	7.1	<0.1	7.14 (0.24)	29 (2.41)	1509 (116.48)	920 (126.21)	16 (1.04)	569 (60.82)	356 (32.74)	3.69	3.4	120.50
II	7.6	1.9	4	0.88 (0.45)	<0.5	513 (390)	463 (32.14)	<0.5	12.2 (1.07)	66 (11.10)	0.04	0.2	0.2
III	6.6	8.2	<0.1	3.12 (0.53)	24 (10.66)	687 (126.6)	1242 (507.05)	16 (5.96)	479 (60)	677 (281.45)	3.8	1.30	174.4
control	6.8	19.5	<0.1	9	<0.5	<5	<0.4	<0.5	<5	<0.4	–	–	–

Table 2. Bioconcentration of zinc, cadmium and lead by earthworms living in collected soils after two exposure times of 24 and 31 days. All values are the average of three replicate samples of ten adult specimens, values in brackets give the SD. Accumulation factor: ratio of bioconcentration (day 31) to total soil concentration

Sample	Zinc (µg/g dry wt)			Cadmium (µg/g dry wt)			Lead (µg/g dry wt)		
	day 24	*day 31*	*accumul. factor*	*day 24*	*day 31*	*accumul. factor*	*day 24*	*day 31*	*accumul. factor*
IA	118 *(20)*	175 *(28)*	0.18	27 *(3)*	34 *(4)*	2.63	93 *21*	99 *(10)*	0.12
IB	191 *(22)*	174 *(3)*	0.03	56 *(17)*	60 *(4)*	0.67	521 *(86)*	534 *(58)*	0.13
IC	168 *(18)*	202 *(81)*	0.22	74 *(5)*	92 *(7)*	3.18	305 *(31)*	327 *(63)*	0.22
II	104 *(10)*	117 *(9)*	0.25	23 *(5)*	25 *(12)*	–	100 *(15)*	81 *(24)*	0.16
III	137 *(12)*	303 *(99)*	0.24	58 *(4)*	66 *(3)*	2.76	93 *(11)*	134 *(5)*	0.19
control	132 *(8)*	132 *(24)*	–	<0.5	<0.5	–	<5	<5	

Lead is also concentrated by worms, but to a lesser extent; the highest accumulation factors were found for IC (0.217) and III (0.196) soil type. Concerning zinc accumulation, the highest accumulation factor was found for II-soil and the lowest for IB-soil. It must be noticed that the soil of site II is the least contaminated, and has a very low organic matter content (1.9 %). At the opposite extreme, IB-soil has a very high OM content (11.6 %) with very high heavy metal contents.

In contrast with lead and zinc, worm cadmium burdens could not be related to soil content using a linear model. After 24 days of exposure, the relationship between the log of worm lead burdens to NAX soil values was significant: $P < 0.01$ (Fig. 1a). Log worm lead burdens related to log NH_4Ac soil values gave a less significant correlation: $y = 0.794\ x - 0.088$, $r = 0.816$ ($P < 0.05$) in contrast to the zinc values. Indeed, the correlation coefficient for log of

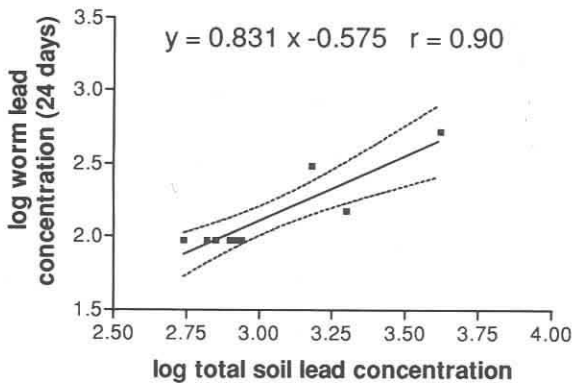

Fig. 1a. Lead concentration in worms after 24 days in contaminated soil, in relation to the nitric acid extractable field soil fractions. Regression equation and 95 % of confidence intervals are given

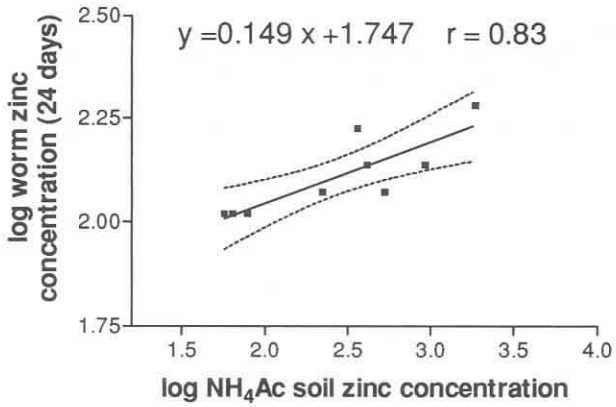

Fig. 1b. Zinc concentration in worms after 24 days in contaminated soil, in relation to the ammonium acetate extractable field soil fractions. Regression equation and 95 % of confidence intervals are given

worm zinc burdens and log NH_4Ac extractable zinc (Fig. 1b): $r = 0.83$ ($P < 0.01$), was higher than for nitric acid extractable metal: $y = 0.227\ x + 1.43$, $r = 0.82$, indicating that worm burdens were more closely related to NH_4Ac extractable zinc than to NAX concentrations. Whatever the reason, the slope parameters for the two relationships with zinc are far below one, indicating that the accumulation of zinc in tissues may be regulated; this phenomenon has previously been observed by Spurgeon & Hopkin (1996b). This was not observed for lead. Concerning $CaCl_2$ extracted heavy metal content, one has to note that lead is under the detection limits for two soil types (IA and II); thus, it is not possible to draw any conclusion from these results. Cadmium extracted by this technique is far below the amount found in the worm tissues; therefore $CaCl_2$ extraction does not provide a reliable estimation of worm cadmium content.

Effect of heavy metals burdens on GST activities

GST activities of worms were measured after 6 weeks of exposure in the different contaminated soils. For comparison, worms living in commercial mould (in which no zinc, cadmium or lead has been detected) were subjected to the same treatment. Mean activities and SD (nkat/g of protein) are shown in Fig. 2. Differences between means of enzyme activities were tested for statistical significance by ANOVA. No significant differences between the control group and heavy metal exposed worms were found.

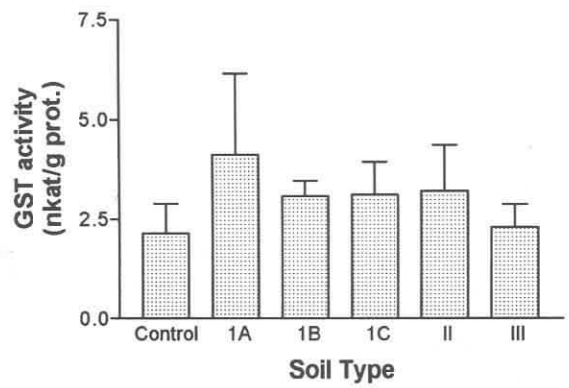

Fig. 2. GST activities (nkat/g prot.) in whole worms *Eisenia fetida* after 6 weeks of exposure to heavy metal contaminated soils. Mean and SD

In vitro effects of zinc, cadmium, lead and copper on GST activities of normal or "contaminated" worms (living and reproducing in artificially polluted soil with 80 mg/kg of $CdCl_2$ for two years) have been measured. Mean activities and SD are shown in Table 3. No significant differences between the control group and worms born and reproducing in cadmium artificially contaminated soil were found. No effect of any of these four metals was observed at these concentrations for any group of worms.

Table 3. Effects of zinc, cadmium, lead, or copper on GST activities (nkat/g prot.) in vitro in whole *Eisenia fetida* according to treatment. Means and SD of four replicates

Control worms					worms living in polluted soil *(80 mg/kg $CdCl_2$)*				
control	zinc	cadmium	lead	copper	control	zinc	cadmium	lead	copper
1.21 *±0.10*	1.21 *±0.18*	1.20 *±0.04*	1.13 *±0.37*	1.10 *±0.20*	1.27 *±0.22*	1.29 *±0.14*	1.23 *±0.42*	1.06 *±0.25*	1.00 *±0.12*

Discussion

Cadmium uptake by earthworms is very efficient and the total burdens of worms are much greater than the Total or NH_4AC or $CaCl_2$ extracted soil content. This result is comparable to that found in other studies concerning different species of worms (Ma et al. 1983; Ireland 1979). Moreover, it was suggested by Neuhauser et al. (1995) that bioconcentration of worms collected in contaminated fields depends on the metal concentration in the soil, and that bioconcentration was greater at lower heavy metal concentrations. Such a phenomenon was observed in our experiments for cadmium uptake from IB-soil: worms living in the most contaminated soil (IB) had a smaller cadmium concentration factor. Nevertheless, other factors may explain the differences observed. Generally, it has been found that solubility and expected availability of contaminants to organisms were influenced by several soil factors such as pH, metal concentrations, organic matter or clay content (Spurgeon & Hopkin 1996b; Abdul Rida & Bouché 1997), zinc and cadmium being more soluble at lower pH and percentage OM values (Crommentuijn et al. 1997). In our experiments, IB-soil had a very high organic matter content (11.6 %) and probably a low availability of metals.

Different chemical extraction techniques of metals have been tried by different authors to compare and evaluate the best expression of these results with earthworm heavy metal body burdens. Abdul Rida & Bouché (1997) observed that, generally, acid acetic or DTPA (dieethylene triamine pentaacetic acid) extracted heavy metal fractions did not give better estimates of assimilable metals by earthworms than did total extraction. $CaCl_2$ extraction, useful for the estimation of availability of metals for plant uptake, cannot be used for estimation of Cu availability (Marinussen et al. 1997), since the 0.01 M extractable heavy metal contents were close to or below the determination limit. We found comparable results for lead. Moreover, Spurgeon et al. (1996b) observed that worm zinc burdens of exposed *Eisenia fetida* were more closely related to water soluble than nitric acid extracted concentration. In our experiments, except for zinc, water extractable contents of cadmium or lead were below the determination limit (data not shown). Water extraction of heavy metals seems to be unreliable (except for high zinc levels) and cannot be used to mimic bioavailability of metals to earthworms.

In conclusion, except for zinc, which seems to be more closely related to less drastic extraction techniques, total soil content of metals seems to be the most interesting technique to predict worm heavy metal burdens.

Worm heavy metal detoxification may involve metallothioneins (Susuki et al. 1980; Yamamura et al. 1981). Glutathione and glutathione-S-transferase system are also important in intracellular protection against toxic metals, oxidative mecanisms or xenobiotic substances. Though inducibility of GST with lead or copper has been found in several vertebrates (Susuki

et al. 1996; Manjeet & Ravindra 1996; Martinez-Lara et al. 1996), we observed no induction effect in the worm *Eisenia fetida typica*. The original function of GST in worms is not yet clear but its non-inducibility, as also for mixed-function-oxygenase system by organic compounds, seems to be a common phenomenon (Milligan et al. 1986; Stokke & Stenersen 1993; Borgerras et al. 1996). It may be suggested that the non-inducibility, compared to herbivorous arthropods or mammals is due to their detritus diet. The ability of enzyme induction has probably evolved as an adaptation to toxic secondary plant metabolites (Brattsten et al. 1977; Yu 1982).

The intrinsic function of GST in worms may be to support excretion of endogenous catabolic products, rather than to support detoxication of xenobiotics (Stenersen 1984). A ligand function of earthworm GST cannot be excluded (Stenersen & Øien 1981). No inhibition of GST by heavy metals was observed in worm extracts, in contrast to what has been observed in vitro in rat livers (Dierickx 1982) . In vivo inhibition of oxidative enzymes by cadmium or lead has been observed in several aquatic invertebrates such as mussels (Prakash & Rao 1995), crayfish (Meyer 1991; Almar et al. 1987) or dragonfly larvae (Meyer et al. 1986). Nevertheless, the authors always notice that these effects are tissue or organ dependent (Almar et al. 1987; Meyer et al. 1991). Moreover, a decrease of enzyme activity in tissue where heavy metals are concentrated has generally been observed. In worm, although heavy metals are essentially concentrated in chloragogenous cells (Ireland & Richards 1981), the level of GST in these cells is low, whereas the higher activity of enzymes is found in the nephridia (Stenersen & Øien 1981). This may explain why no effect can be observed in vivo or in vitro on the whole worms.

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